*2009 International Nuclear Atlantic Conference - INAC 2009*
*Rio de Janeiro,RJ, Brazil, September27 to October 2, 2009*
*ASSOCIAÇÃO BRASILEIRA DE ENERGIA NUCLEAR - ABEN*
***ISBN:* 978-85-99141-03-8**

# USING LANTHANIDE CHELATES AND URANYL COMPOUNDS FOR DIAGNOSTIC BY FLUOROIMMUNOASSAYS

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## ABSTRACT

The importance of the luminescence of lanthanide ions and $UO_2^{2+}$ is related to its peculiar characteristics, e.g. long lifetime and line-like emission bands in the visible, which make these ions unique among the species that are known to luminescence. Recent developments in the field of supramolecular chemistry have allowed the design of ligands capable of encapsulating lanthanide ions, thus forming kinetically inert complexes. By introduction of chromophoric groups in these ligands, an intense luminescence of the ion can be obtained via the "antenna effect", defined as a light conversion process involving distinct absorbing (ligand) and emitting (metal ion) components. In such a process, the quantities that contribute to the luminescence intensity are (i) the efficiency of the absorption, (ii) the efficiency of the ligand-metal energy transfer, and (iii) the efficiency of the metal luminescence. Encapsulation of lanthanide ions with suitable ligands may therefore give rise to "molecular devices" capable to emit strong, long-lived luminescence. Besides the intrinsic interest in their excited state properties, compounds of lanthanide ions, in particular of the $Eu^{3+}$ and $Tb^{3+}$ ions, and now $UO_2^{2+}$ are important for their potential use as luminescent labels for biological species in fluoroimmunoassays (FIAs). This is most interesting because fluorimetric labeling represents an alternative method to the use of radioactive labels, which has long been the most common way of quantifying immunoreactions. In this article we report information about luminescent materials, which gave a good signal to quantify biological molecules by TR-FIA, DELFIA , DSLFIA, RIA and FRET.

## 1. INTRODUCTION

### 1.1 Luminescent materials

Luminescent materials, especially lanthanides, are applied in many devices of importance [1]. The interest in the photophysical properties of lanthanide complexes which act as optical centers in luminescent hybrid materials has grown considerably since Lehn [2] asserted that

such complexes could be seen as light-harvesting supramolecular devices. Particularly, the design of efficient lanthanide complexes has acquired the attention of many research groups, focusing on the diverse classes of ligands, β-diketones, heterobiaryl ligands, etc. An alternative and very attractive possibility in regard to the complexation of lanthanide ions using ligands that are covalently fixed to the inorganic networks has emerged. To date, few studies in terms of covalently bonded hybrids with increasing chemical stability have appeared and the as-derived molecular-based materials exhibit monophasic appearance even at a "high concentration" of lanthanide complexes [3,4].

There is a greater interest in complexes of $TR^{3+}$, WEISSMAN since discovered that the excitation in these compounds can be made under acceptable conditions, light absorbed by the ligands with subsequent transfer of energy to the metal center [5]. The transfer of energy from the organic chromophore to the rare earth ion provides an effective way to excite the emission of fine and long life-span. The direct excitation of $TR^{3+}$ ions is difficult because of the nature of forbidden electronic transitions in these ions. One of the most important applications of rare-earth complexes is as luminescent markers in clinical diagnosis where they are an alternative to radioactive probes [6-8].

Among the rare-earth compounds which exhibit luminescence, the most studied are the ions $Eu^{3+}$ and $Tb^{3+}$ (light emitting red and green, respectively), which presented higher luminescent intensity in the visible, due to the structures of their energy levels. One must consider also that there are compounds of $TR^{3+}$ broadcast in other spectral regions such as near infrared ($Yb^{3+}$, $Nd^{3+}$ and $Er^{3+}$), orange ($SM^{3+}$), yellow ($Dy^{3+}$), blue ($Tm^{3+}$) and near-UV ($Ce^{3+}$ and $Gd^{3+}$) [9]. These complexes are promising luminescent because their actions as markers in cytology and immunology, which can be used as luminescent biomarkers [10]. They have also shown potential to also act as potential drugs for the treatment of cancer [11].

In 1978, Gutsche et al. synthesized a series of macrocyclic phenol formaldehyde condensates with the given name calixarenes and the molecular receptor activity is now their most significant property [12]. The successive search for new synthetic molecular receptors capable of guest-host relationships with metal ions and neutral molecules has triggered lots of novel structures during the past 20 years [13]. Macrocyclic as calixarenes, with or without the addition of molecular groups to act as an antenna are able to transfer energy to the lanthanide ion $Ln^{3+}$ under excitation at the energy appropriate. Currently great interest in sensors for lanthanide ions and to FIA's has been demonstrated, but some of the tasks required are: the complex must be stable, which has the high molar absorptivity and the cation has to be shielded from the effects of suppression caused by the solvent and increase the lifetime of the excited state. Although calixarenes form stable complexes with the f elements few studies have been published in this field and a design of ligands calixarenes to meet the needs of the factors governing the selection of groups are not fully understood or resolved [14].

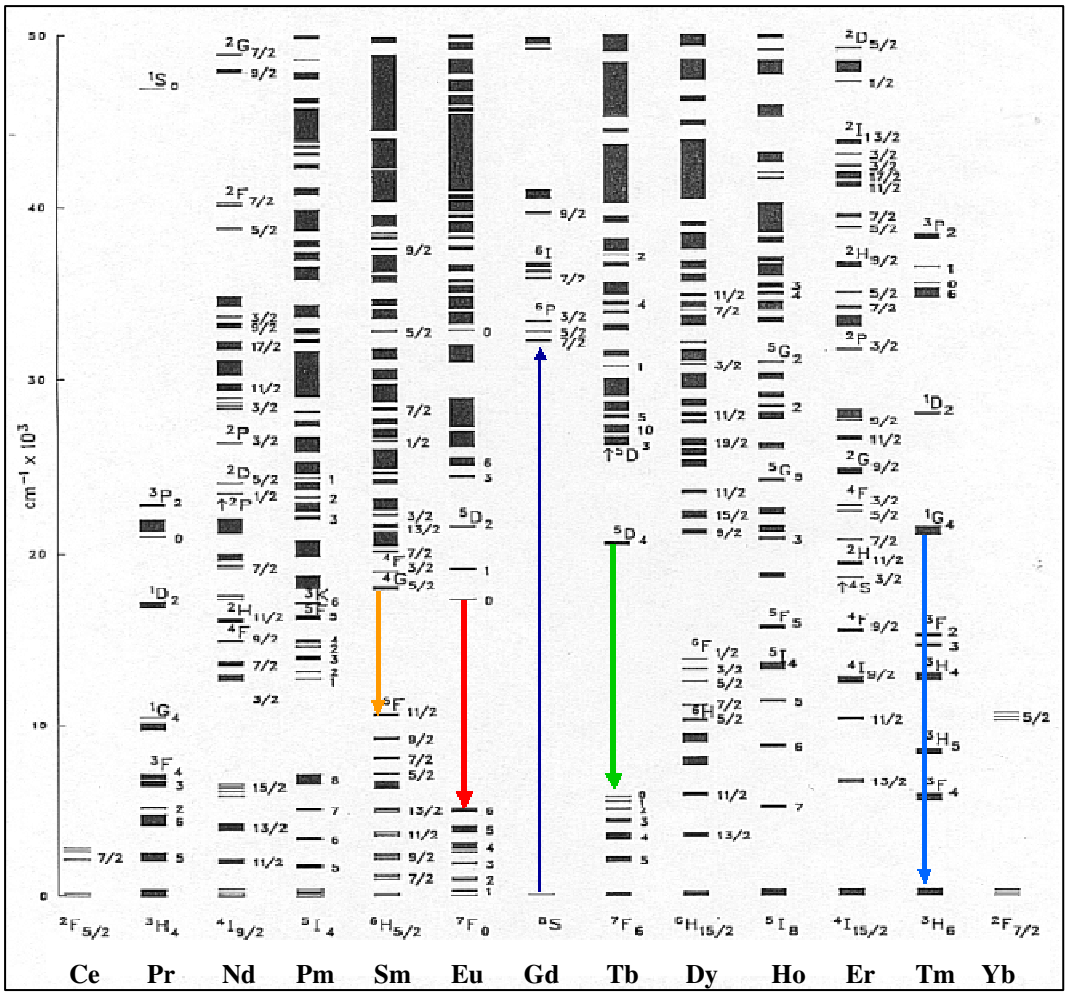
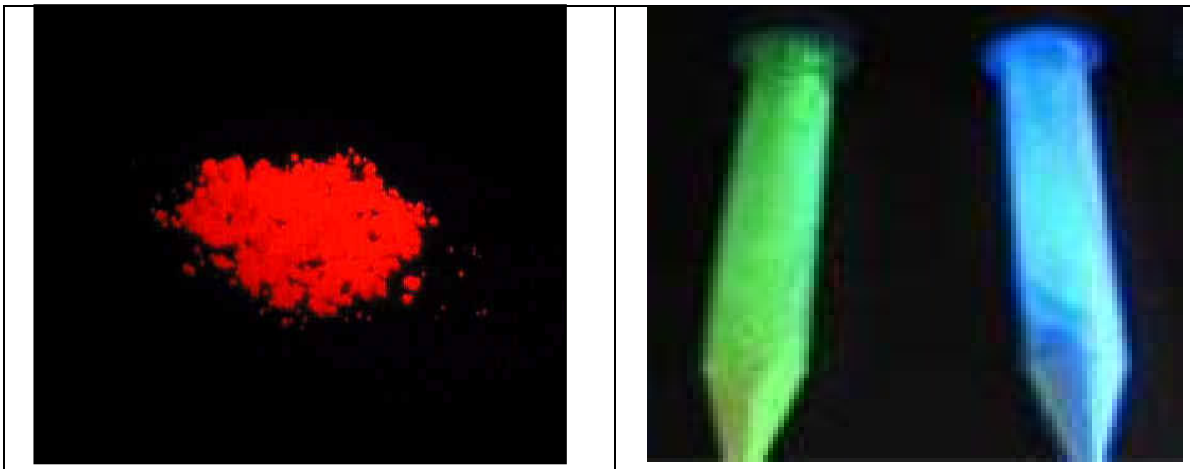



**Figure 1. Structure of lanthanide ions energy levels based on the crystal field .**



**(a)** **(b)**

**Figure 2. (a) $Eu^{3+}$ and (b) $Tb^{3+}$ e $Tm^{3+}$ excited by ultraviolet radiation.**

The uranyl ion has great potential as a luminescent material, applied in laser technology, luminescent probes, cells to convert solar energy, etc [15]. The main focus of studies of salts of uranium and its complexes is to determine the types of interactions between the ion and its ligands. In hydrated salts, usually the removal of water molecules does not affect significantly the fluorescence. However, it is known that the water molecules adjacent to the uranyl ion affect the lifetime of fluorescence. The removal of these molecules of water or the exchange of other ligands generally rises this lifetime. The luminescence of compounds containing lanthanide ions and $UO_2^{2+}$ has been increasing among the optical properties of these ions that thanks to the peculiar characteristics such as time and life-long thin strips of issue in the UV-visible [16].

The search for a sensitizer for europium ion is important because it shows the absorption bands of low intensity. In a more specific you can cite the studies of Kropp transfer of power [17] in which he shows that there is transfer of energy in a short range of the spectrum of $UO_2^{2+}$ to $Eu^{3+}$ in aqueous solution of perchlorate, suggesting the strong influence of the complex oligomers hidroso , probably containing bridges between the $OH^-$ ion $UO_2^{2+}$ and $Eu^{3+}$ . The transfer of energy from the group $UO_2^{2+}$ to the $^5D_0$ level of $Eu^{3+}$ was observed in phosphate-based glasses [18].

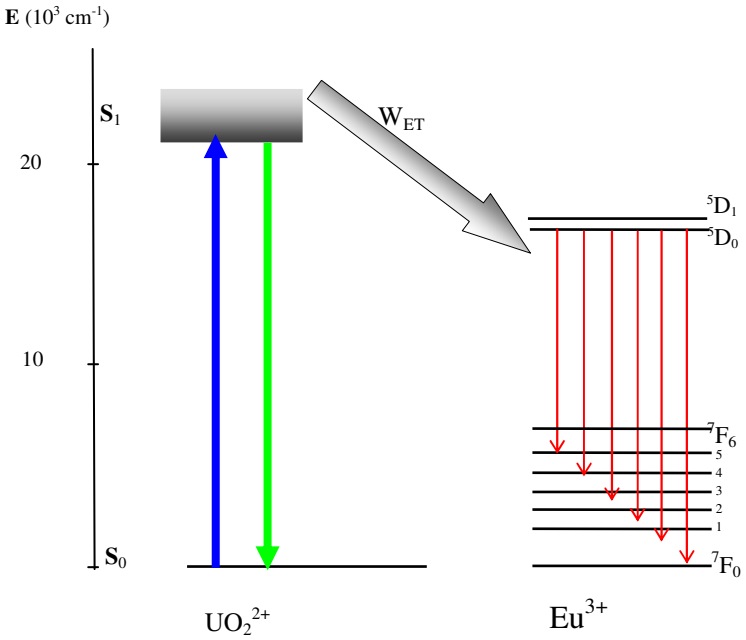



**Figure 3. Schematic diagram of energy levels for ions $UO_2^{2+}$ and $Eu^{3+}$ and processes of excitation, emission and transfer of energy.**

Uranium minerals have drawn the attention of scientists due to their hazardous nature and their commercial importance. Among the different analytical techniques involved in the study of uranium containing minerals, micro-Raman spectroscopy has been intensively applied due to the ease of operation and fast assessment of their molecular characteristics [19]. In an aqueous environment exposed to air, the most stable species of U is uranyl ion $(UO_2)^{2+}$ [20].

Coordination compounds with uranyl ion and lanthanide ions in the same molecular unit are extremely rare, and the first examples, obtained under hydrothermal conditions with 1,4,7,10-tetraazacyclododecane-N,N′,N″,N‴-tetraacetic acid (DOTA) and oxalato ligands, were reported only very recently [21]. For example, the lanthanide ion (Gd or Eu) occupies the $N_4O_4$ site of the macrocycle, as usual, and the uranyl ion, bound to carboxylate oxygen atoms directed outward and to oxalate groups, connects the $[Ln(DOTA)(H_2O)]^-$ units to form a two-dimensional framework.

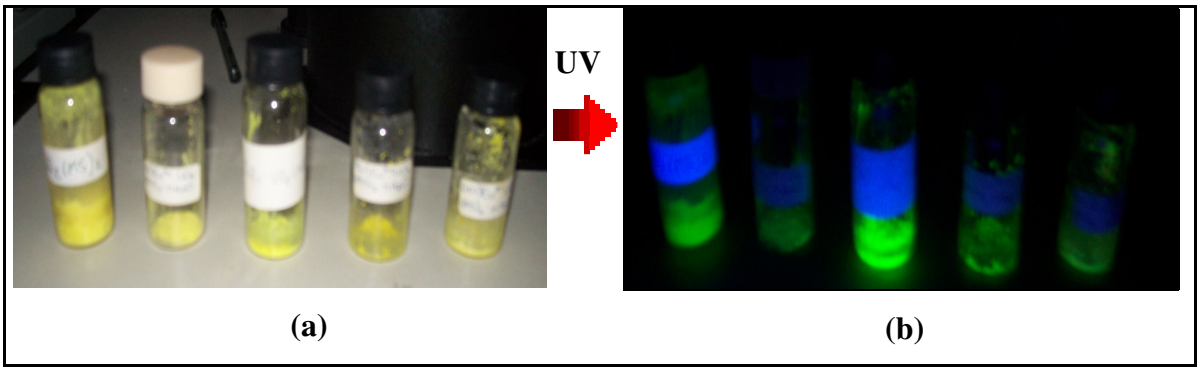



**Figure 4. (a) Uranyl matrices obtained in this group work and (b) the same matrices exposed to ultraviolet radiation.**

## 1.2 Ligands

Ligands that do not display two different coordination sites seem at first less suitable for the complexation of both of these cations, which present very different coordination geometry requirements (equatorial for uranyl and spherical for lanthanides). Attempts to use various polycarboxylic acids, for example, consistently give crystalline solids containing only the uranyl ion. Cucurbiturils (CBs) may not appear very promising to achieve this particular heterometallic coordination, although their lanthanide complexes have been much investigated, and several uranyl complexes were recently described [22].

The coordination compounds β-diketones most studied are those involving the complexes containing ligands because of their skills of coordination are well established [23]. Several types of ligands can form adducts with the chelates of $TR^{3+}$-tris(β-diketones) among them: sulfoxide, dimethylformamide, triphenylphosphine oxide, and which has heterocyclic atoms of nitrogen (donor), such as pyridine, 2,2'-dipiridil, 1,10-phenanthroline 2,2': six', 2''-terpiridina, among others. In order to obtain highly luminescent materials, have prepared the salts of β-diketones (ex: TTA and DBM) of rare earth hydrated, with subsequent replacement of water molecules by ligands (sulfoxide, fosfinóxidos and amides). These ligands coordinated to the ion $TR^{3+}$ allowed better efficiency of energy transfer in ligand-metal ($L \rightarrow TR^{3+}$). This coordination usually involves the called antenna effect, so absorption of ultraviolet light incident the organic part, efficiently transferring energy to the luminescent center ($TR^{3+}$ ions) and subsequent issue [24-27].

Lanthanide complexes, with extremely long luminescence lifetimes in the millisecond range, are ideally suited for biomedical diagnosis applications [28]. Several luminescence based probes and sensors for copper with limits of detection within the nanomolar concentration range have been reported over the past decade. Among them, sensors based on Lucifer Yellow [29] embedded in an anion exchanger hydrogel, FRET sensors [30] and fluorophores entrappedin PVC [31, 32] were used for $Cu^{2+}$ detection. Furthermore, biomolecules such as red fluorescent protein (DsRed) [33, 34] or carbonic anhydrase coupled to commercially available fluorophores [35] were found to selectively bind $Cu^{2+}$.

The use of lanthanide chelates as luminescent indicators, rather than conventional fluorophores, can enable highly sensitive detection due to their specific properties. In particular, the large Stokes' shift of lanthanide chelates (mostly $Eu^{3+}$ and $Tb^{3+}$) easily permits selection of the chelate-specific emission from scattered excitation light, even with filters. The narrow emission bands allow efficient separation of several luminescence signals in multicolor assays. Further, the very long luminescence lifetime permits gated detection on a micro to millisecond timescale, to avoid typical short-lived non-specific background signals [36, 37]. In these systems, intense ion luminescence originates from the intramolecular energy transfer from the excited triplet-state of the ligand to the emitting level of the lanthanide (antenna effect) [38].

### 1.3 Fluoroimunoassay

The development of novel methods for rapid, sensitive and low-cost immunoassays attracts particular interests because of their wide applications in medical diagnostics, food inspection and biomedical research [39]. Conventional techniques such as enzyme linked immunosorbent assay (ELISA) and radial immunodiffusion (RID) are suboptimal for protein detection, considering the specific storage of active enzymes in ELISA and potential health hazard of radioisotopes in RID [40, 41]. To improve the detection sensitivity and/or to shorten the detection time, many immunosensors have been fabricated recently using different principles, such as fluorescence [42], electrochemistry [43], Raman [44], etc. Most of these methods require complicated structures, delicate manipulation or sophisticated instrumentation, which motivates the exploitation of new immunosensors with simple operation and high sensitivity.

The use of lanthanide elements in time-resolved fluoroimmunoassays (TR-FIA) opened a new era in fluoroimmunoassays (FIA). Up to now, though dissociation enhanced lanthanide fluoroimunoassay (DELFIA) has been commercialized [45], at a high sensitivity; it still has some limitations on the release of $Eu^{3+}$ from the chelate, and can not produce a specific space signal. This restricts its applications in some important areas, such as fluorescence imaging, immunohistochemistry, localization hybridization and online detection.

TR-FIAs have been utilized in the analysis of veterinary drugs [46, 47]. In general, TRFIA can offer better sensitivity than the more traditional ELISAs due to the unique fluorescent properties of the lanthanide chelates and the time-resolved measurement mode, which enable the specific fluorescence to be measured after the background fluorescence has already

declined [48]. TR-FIA is one of the fastest growing non-isotopical immunoassay technology [49, 50]. The most important advantage of time-resolved fluorometric measurement using lanthanide fluorescence chelate as the label is that the specific fluorescence signal from long-lived labels and the background noise from biological samples and solid-phase materials can be easily distinguished. In the past several years, one important development of TR-FIA was homogeneous TR-FIA based on the fluorescence resonance energy transfer (FRET) between a lanthanide fluorescence label and an organic fluorescence dye, in which, the complicated operations of B/F separation (separation of bound reagent and free reagent) and solid-phase carrier in the conventional immunoassay methods are not necessary.

What is more, another limitation of this technique is that the assay precision may easily be deteriorated due to the easy contamination of the rare earth ion from the enhancement solution. To avoid these problems, in 1987, Evangelista and Diamandis synthesized 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA) and studied auxiliary apparatus systems. They adopted them into time-resolved solid-phase fluoroimmunoassays (DSLFIA) [51, 52], where an enhancement solution was not necessary and the concentration of antibody or antigen could be directly measured by laser excitation on solid-phase. Thus, this made the operation of TR-FIA simple, quick and much easier.

Meanwhile, its application range was also extended. However, there still existed something unsolved. For example, the fluorescence was weaker than that in the DELFIA system; how to prepare the label with high relative immunological activity and high fluorescence intensity was not clear; storing conditions of BCPDA and the methods of measuring relevant parametern of the label were troublesome. Later, an intense and steady fluorescence emission was observed in promising lanthanide chelates [53], when some cage-type chelates were used in immunoassays, but relative large particle sizes and surface character brought a certain number of questions in storing and applications. In addition, other new chelates were also reported by Ci and other authors [54, 55]. Nevertheless, as a directly luminescent lanthanide chelate for DSLFIA, some factors still remain open, such as the luminescence intensity, solubility and stability. Therefore, research on DSLFIA is still needed.

Nanoparticle (NP)-based assays have been extensively used for chemical and biological detection. As a carrier for encapsulation of many quantum dots or dye molecules, NPs have been mainly used as signal reporters in fluoroimmunoassays (FIA) to improve the detection sensitivity [56, 57]. Although thousands of fluorescent molecules could be encapsulated into NP matrix, the potential application of NP-based biomarkers is limited due to the leakage of fluorescent molecules in washing steps [58]. In addition, dye-doped NPs often suffer from broad emission and small Stokes shift, which could result in cross-talking between excitation and emission signals [59]. To improve signal output and minimize cross-talking, one efficient method is to introduce fluorescence resonance energy transfer (FRET), which separates the wavelengths between the excitation of the donor and the emission of the acceptor.

Fluorescence resonance energy transfer from cationic conjugated polymers (CCP) to dye is a prerequisite for high signal amplification, which is determined by donor/acceptor distance, spectral overlap between the donor emission and acceptor absorption, and orientation factor

according to Förster equation [60]. In addition, other factors, such as well-matched energy levers of donor–acceptor pairs, charge density of the donor, and solvent media were also found to affect the signal amplification. Water-soluble CP-based sensors that operate on FRET mechanisms have been widely used to detect nucleic acids and small molecules in solution and on solid substrate [61]. So far, there is no direct demonstration that water-soluble CPs could be used to amplify the signal from dye labeled antibodies or other proteins due to the structure diversity and complexity of the target analytes.

Radioimmunoassays (RIAs) have been used to measure the concentration of various hormones due to their high sensitivity and accuracy. However, RIAs require handling of radioisotopes and can only be carried out in designated areas. In contrast, time-resolved fluoroimmunoassays (TR-FIAs) using lanthanide-labeled compounds are carried out without the need for special facilities and can attain a high sensitivity equal to that of RIAs. Moreover, the labeling procedure of peptide hormones with lanthanides is not complicated, and the labeled compounds can remain stable for a long time at –20°C. There are significant correlations between data from TR-FIA and RIA [62], and TR-FIAs have been used to measure hormone concentrations in the blood and organs of several species [63, 64].

In a time-resolved amplified cryptate emission (TRACE) homogeneous TR-FIA system developed by Mathis and coworkers [65], two kinds of fluorescence molecules, $Eu^{3+}$–trisbipyridine cryptate [TBP–$Eu^{3+}$] and cross-linked allophycocyanin (named XL665 by Mathis et al.) were employed as the donor–acceptor pair of FRET. Since the fluorescence lifetime of the donor label [TBP–$Eu^{3+}$] is rather long (1ms), the lifetime of sensitized emission by FRET at 665 nm from the acceptor label, XL665, is increased to 0.25ms. By time-resolved fluorometric measurement at 665nm, the signals from the acceptor in the immune complex and the excess donor and acceptor in the solution were distinguished clearly. More recently, homogeneous TR-FIA was further developed by using an $Eu^{3+}$ fluorescent chelate and Cy5 (small organic molecule) or a $Tb^{3+}$ fluorescent chelate and tetramethylrhodamine as donor–acceptor pairs, and applied for the measurements of the IL-2 and IL-2R (interleukin) reaction [66] and the subunit of human chorionic gonadotropin [67] in serum.

## 2. CONCLUSIONS

The review showed that NP-based fluoroimmunoassay for detection is simple and sensitive and the addition of energy provides signal amplification via FRET, resulting in a fluoroimmunoassay with high detection sensitivity. This strategy could also be generalized for other assay format for different protein detection with simple operation and high performance. The covalently bonded hybrid materials present longer lifetimes than the doped hybrid ones; especially, the luminescent quantum efficiency of covalently europium hybrids is higher than that of the doped europium one. The existence of coordination water molecules to lanthanide ions produces severe nonradioactive energy loss to decrease the luminescent properties. These informations serve to illustrate the potential of ligands with regard to constructing interesting supramolecular structures and for purposes of incorporating

predictable physical properties. From a more general perspective, the coupling of proteins and luminescence in a material has interesting prospects for the development of luminescence materials. TR-FIA method is sensitive and stable immunoassay as a rapid screening method could be applied to routine residue analysis.

## ACKNOWLEDGMENTS

The authors are grateful to the Comissão de Energia Nuclear (CNEN) for financial support, Rede de Nanotecnlogia Molecular e Interfaces (RENAMI) and Instituto Nacional de Ciência e Tecnologia de Nanotecnologia para Marcadores Integrados (INCT-INAMI). The authors would also like to thank IPEN`s Laboratories.

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